Sanitizing the fortress: protection of ant brood and nest material by worker antibiotics

Article (Accepted Version)

Sanitizing the fortress: protection of ant brood and nest material by worker antibiotics

C. TRANTER[1,2], P. GRAYSTOCK[1], C. SHAW[1], J. F. S. LOPES[3] and W. O. H. HUGHES[2]

[1]*School of Biology, University of Leeds, Leeds, LS2 9JT, UK*

[2]*School of Life Sciences, University of Sussex, Brighton. BN1 9QG, UK*

[3] *Pós-Graduação Comportamento e Biologia Animal, Instituto de Ciências Biológicas, Campus Universitário de Martelos, 36.036-330, Juiz de Fora, MG, Brazil*

Correspondence: Christopher Tranter, School of Life Sciences, University of Sussex, Brighton. BN1 9QG, UK; e-mail: c.tranter@sussex.ac.uk

Abstract

Social groups are at particular risk from parasite infection, which is heightened in eusocial insects by the low genetic diversity of individuals within a colony. To combat this, adult ants have evolved a suite of defences to protect each other, including the production of antimicrobial secretions. However, it is the brood in a colony that are most vulnerable to parasites because their individual defences are limited, and the nest material in which ants live is also likely to be prone to colonisation by potential parasites. Here we investigate in two ant species whether adult workers use their antimicrobial secretions not only to protect each other, but also to sanitize the vulnerable brood and nest material. We find that in both leaf-cutting ants and weaver ants, the survival of brood was reduced, and the sporulation of parasitic fungi from them increased, when the workers nursing them lacked functional antimicrobial-producing glands. This was the case both for larvae that were experimentally treated with a fungal parasite (*Metarhizium*) and for control larvae which developed infections of an opportunistic fungal parasite (*Aspergillus*). Similarly, fungi were more likely to grow on the nest material of both ant species if the glands of attending workers were blocked. The results show that the defence of brood and sanitization of nest material are important functions of the antimicrobial secretions of adult ants, and that ubiquitous, opportunistic fungi may be a more important driver of the evolution of these defences than rarer, specialist parasites.

Keywords: parasite, social immunity, social insect, disease resistance, metapleural gland, venom gland, nest hygiene, *Metarhizium, Aspergillus*

Introduction

Many species form social groups, and by doing so benefit from greater resource exploitation, anti-predator defence and reproductive fitness (Dornhaus et al. 2010). However, such benefits come at the potential cost of increased parasite exposure (Alexander, 1974; Krause and Ruxton 2002). Eusocial insects are one of the pinnacles of sociality, but their vulnerability to parasites is heightened by a homeostatic nest environment and low genetic diversity of individuals within a colony, which will facilitate parasite transmission and evolution (Schmid-Hempel 1998). To counter this, social insects, such as ants, have evolved a suite of behavioural and chemical defences which physically remove or chemically kill parasites that contaminate their cuticle (Boomsma et al. 2005; Wilson-Rich et al. 2009). These first-line defences are important for resistance to specialist entomopathogens and also the more common opportunistic parasites which abound in and around ant colonies (Milner et al. 1998; Schmid-Hempel 1998; Poulsen et al. 2006; Evans et al. 2011; Fountain and Hughes 2011; Reber and Chapuisat 2012; Andersen et al. 2012). Ants (Storey et al. 1991; Mackintosh et al. 1995; Zelezetsky et al. 2005; Mendonça et al. 2009), bees (Evans et al. 2006; Baracchi and Turillazzi 2010; Baracchi et al. 2011), wasps (Turillazzi et al. 2006; Baracchi et al. 2012), termites (Rosengaus et al. 2000, 2004) and eusocial thrips (Turnbull et al. 2011, 2012), as well as non-social insects (Bulet et al. 1999; Kuhn-Nentwig 2003; Haine et al. 2008; Stow and Beattie 2008), produce defensive compounds in their haemolyph and venom. In particular, most ants secrete antimicrobial compounds from their metapleural or venom glands on to their cuticle (Hölldobler and Wilson 1990). The secretions from both glands have been shown to inhibit the growth of parasites *in vitro* and adult workers with non-functional glands are more susceptible to parasites (Storey et al. 1991; Blum 1992; Bot et al. 2001; Poulsen et al. 2002; Graystock and Hughes 2011; Tragust et al. 2013).

Social insects are characterised by cooperation, with workers acting to maximise the fitness of their colony in spite of costs to themselves on an individual level. As a result, the resistance of social insects to disease consists of individual immunity and group-level responses that produce a form of 'social immunity', that can be adaptive and proactive (Rosengaus et al. 1998; Traniello et al. 2002; Hughes et al. 2002; Cremer et al. 2007; Chapuisat et al. 2007; Ugelvig and Cremer 2007; Walker and Hughes 2009; Morelos-Juárez et al. 2010; Reber et al. 2011; Hamilton et al. 2011; Konrad et al. 2012). Social immunity may be particularly important for the more vulnerable aspects of a colony, such as developing brood and nest substrates. Insect brood lack a fully developed physiological immune system (Gillespie et al. 1997; Lavine and Strand 2002; Wilson-Rich et al. 2008), are unable to self-groom, and do not have the important antimicrobial-producing glands (Hölldobler and Wilson 1990). Brood are thus extremely susceptible to disease and may consequently be particularly reliant on social immunity, including potentially the donation of antimicrobial secretions by adult workers. In an elegant study, Tragust et al. (2013) showed that nursing adult workers of *Lasius neglectus* donate venom to brood during grooming, both directly via the acidopore and indirectly through oral uptake, and that this then benefited brood defence against parasites. In addition to brood, the substrate in, on, or with which, ants form their colony is also likely to be vulnerable to contamination, or in some cases infection, by potentially dangerous parasites (Currie et al. 1999; Keller et al. 2003; Hughes et al. 2004; Fountain and Hughes 2011; Reber and Chapuisat 2012). This is particularly evident in the attine fungus-growing ants, which cultivate a mutualistic fungal crop that forms the central substrate of the colony and which is very vulnerable to infection by parasites (Mueller et al. 1998; Currie et al. 1999; Gerardo et al., 2006; Little et al. 2006). Consequently fungus-growing ants will mechanically groom their gardens to remove potential threats, have large metapleural glands and apply metapleural secretions onto the fungal crop (Currie and Stuart

2001; Sumner et al. 2003; Fernández-Marín et al. 2006, 2009; Little et al. 2006; Hughes et al. 2008, 2010). It is likely, therefore, that care, particularly the use of antimicrobial secretions, by worker ants is important to keep colony nest material hygienic.

Here we use the entomopathogenic fungus *Metarhizium anisopliae* with a leaf-cutting ant and a weaver ant to test experimentally if, and how effectively, the antimicrobial secretions produced by the venom and metapleural glands of adult workers are utilised to aid in brood survival, and how worker secretions may be used to keep nest material hygienic.

Methods

We studied two ant species: 1) the Brazilian leaf-cutting ant *Acromyrmex subterraneus subterraneus*, which has large antibiotic-producing metapleural glands (de Souza et al. 2006) as well as a venom gland, and 2) the south-east Asian weaver ant *Polyrhachis dives*, which lacks the metapleural gland but produces venom with antimicrobial properties (Zenghe 1986; Graystock and Hughes 2011). In both species, the respective glands (metapleural and venom) have been shown to be important in the disease resistance of adult workers (Poulsen et al. 2002; Graystock and Hughes 2011). Workers and brood were collected from two colonies of weaver ants (Pd0701, Pd0704) and three colonies of leaf-cutting ants (As085 As086 and As0811) that had been maintained in the lab at 26°C and 80% RH for > 6 months prior to use and showed no apparent signs of decline or infection. Due to the availability of brood at the time of the experiment, all leaf-cutting ant brood were pupae of approximately 5 mm in length, while all weaver ant brood were larvae of approximately 5 mm length. For each species, adult workers were selected of similar size (6-8 mm), cuticle melanisation and location in the colony (and thus inferred age; Armitage and Boomsma 2010). We confirmed in a preliminary experiment that workers of these sizes and ages successfully cared for brood

over 14 days when kept in isolation (i.e. a single ant with a single pupa or larva). As our experimental parasite we used a strain of the entomopathogenic fungus *Metarhizium anisopliae* (isolate 144467, CABI; isolated from the soil of a maize field in Canada) which was exotic to both of the ant species. Fungal conidia were harvested from freshly sporulating media plates, and viability was confirmed to be > 92% throughout the experiments by plating the conidia solutions onto Sabouraud dextrose agar plates, incubating for 24 h and quantifying conidia germination. We applied 0.5 µl doses of species-specific concentrations of conidia in Triton-X, that we had determined in preliminary trials caused 50% mortality to brood (weaver ant: 1 x 10^5 conidia/ml; leaf-cutting ant: 1 x 10^4 conidia/ml).

Experiment 1: Brood care

To determine the importance of adult worker antimicrobial secretions for brood survival, we collected 120 leaf-cutting ant workers and 160 weaver ant workers, split into two cohorts. The leaf-cutting ant cohorts were each formed of 60 ants, with 20 ants from each of the three colonies, whilst the weaver ant cohorts consisted of 80 ants, with 40 ants from each of the two colonies used. Half the ants from each colony had their main antimicrobial-producing glands (the metapleural gland in leaf-cutting ants and venom gland in weaver ants) blocked using nail varnish, and the remaining workers had nail varnish applied to the pronotum as a control (Poulsen et al., 2002; Graystock and Hughes, 2011). After 24 h, we collected 60 leaf-cutting ant pupae and 80 weaver ant larvae, for each of the two cohorts, and surface-treated half of them with the *Metarhizium* parasite and the other half with 0.5 µl of a 0.05% Triton X control solution using a micropipette. Each pupa or larva was then placed in a pot (40 mm diameter) with a single 'nurse' worker ant from the same colony to give four combinations of infected/uninfected brood and workers with functional/non-functional glands, in a full factorial design, with a total of 30 leaf-cutting ant and 40 weaver ant replicates of each (Fig.

S1). Ants were maintained in the pots with moistened cotton wool to supply water and sucrose solution *ad libitum*. Any workers which died during the experiment were replaced with an identically-treated worker. The survival of the brood was monitored for 14 days. Dead brood were each placed on moistened filter paper in a Petri dish at 26°C and 80% RH, and checked daily for the appearance of fungal conidia and conidiophores diagnostic of a *Metarhizium* infection. In order to confirm that the blockage treatment did not affect normal brood-care behaviours, we also compared the behaviour of nurse workers for 20 ants of each species . Half the ants in each species had their respective glands blocked and the other half had the control treatment applied to the pronotum. The ants were placed in a Petri dish with a single item of brood (pupae for leaf-cutting ants and larvae for weaver ants) and a) the duration of any non-grooming interaction between nurse and brood (e.g. carrying, antennation), b) the frequency of physical contact between nurse and brood, and c) the frequency of brood-grooming by the nurse ant, was recorded for a 10 minute period.

Experiment 2: Nest hygiene

Sixty weaver ants (30 ants per colony) were collected from within the nest. Half of the ants from each colony had their venom gland blocked with nail varnish and half had a control treatment on the pronotal spines, for a total of 30 replicates per treatment. One hundred and twenty leaf-cutting ant workers (40 ants per colony) were collected from the outer surface of the fungal crop. The ants from each colony were divided evenly into the four blockage treatments as follows: i) varnish applied to the pronotal spines as a control, ii) metapleural gland blocked, iii) venom gland blocked, or iv) both venom and metapleural blocked, with a total of 30 replicates per treatment. Each ant was placed in a pot with a 10 mm^2 section of either the silk nest material of weaver ants or the fungal garden of leaf-cutting ants, from their

original nest, and balls of cotton wool moistened with water and sucrose solution at 26°C and 80% RH. Thirty further 10 mm^2 sections of nest material were set up identically for each species except no ant was placed in the pot (Fig. S2). The nest substrate was monitored for 15 days for the appearance of any foreign fungus and death of the fungal crop. If a worker died during the experiment then it was replaced with an identically treated worker.

To identify the fungi which developed in the leaf-cutting ant trials, three representative samples of each fungal morphotype (based on external morphology, spore structure, and colour) were isolated on malt extract agar (MEA) plates at 30°C until the fungi produced conidia, and then stored at 4°C. DNA was extracted from the samples by adding 200 µl of 5% Chelex solution (in 10 mM Tris buffer) and 0.05 g of 0.1 mm silica beads to approximately 0.05g of the sample fungus, and placed in a QIAGEN Tissue Lyser beadbeater for 4 min at 50 oscillations/s. Samples were then incubated at 90°C before being centrifuged for 30 min at 4°C. Supernatant from the samples was cleaned with OneStep-96 PCR Inhibitor Removal Kit (Zymo Research) prior to PCR amplification of the internal transcribed spacer regions 1 and 2 with the primers ITS1 and ITS4 (Henry et al. 2000; Foley et al. 2012). PCR products were sequenced and fungi identified by BLASTn searches of the resulting sequences.

Statistical analysis

The effects of *Metarhizium* exposure, gland closure, and ant species, on brood survival, and the effects of gland closure and ant species on the appearance of foreign fungi on nest material, were analysed using Cox proportional-hazards regression models. Colony-of-origin and cohort (in Experiment 1), were included in the models to account for the structured nature of the data. Pairwise Kaplan-Meier tests were used to test for pairwise differences

between treatment groups. The effects of blockage on the duration of behavioural interactions of nurse ant and brood were examined using Mann-Whitney U-tests, and the survival of the nurses analysed using Cox proportional-hazards regression models. The proportions of brood sporulating with fungi were examined with χ^2 tests and the proportions of nest material sporulating with fungi were analysed with Fisher's exact tests.

Results

Experiment 1: Brood care

Workers of both species tended to the brood throughout the experiment and the survival of brood that were cared for by a replacement worker did not differ from those that were cared for by the same worker ant throughout (leaf-cutting ants: Wald=2.54, p=0.111; weaver ants: Wald=0.19, p=0.67). Nurse worker ants with blocked or unblocked glands did not differ in their behaviours when attending to brood or in their survival throughout the experiment; Fig. S3. There were significant effects of both exposure to *Metarhizium* and of gland blockage on brood survival, (Wald=17.8, p<0.001; Wald=27.2, p<0.001, respectively), but no overall difference between the ant species (Wald=1.84, p=0.1), or significant interactions between these effects (p >0.2 in all cases). There was no difference in brood survival between leaf-cutting ant cohorts (Wald=0.54, p=0.817), but mortality was higher in the second, compared with the first, cohort of weaver ants tested (Wald=8.52, p=0.004), and there were no significant differences between colonies (P>0.1 in both species). In both ant species, gland blockage reduced brood survival regardless of treatment, while the effect of *Metarhizium* exposure was less consistent (Fig. 1). Compared to the control brood cared for by nurse ants with functioning glands, the hazard ratio for the leaf-cutting ant brood was increased to 2.7 by blocking the metapleural gland, to 3.7 by exposure to *Metarhizium* when the metapleural

gland was functional, and to 5.5 by both exposure to *Metarhizium* and blocking the gland. For the weaver ant brood, the hazard ratio was increased to 1.9 by exposure to *Metarhizium* with the venom gland of nurse ants functional, to 3.4 by blocking the venom gland, and to 4.7 by both exposure to *Metarhizium* and blocking the gland.

Significantly fewer of the *Metarhizium*-exposed weaver ant brood sporulated with *Metarhizium* when the venom glands of their nurse ants were functional than when the glands were blocked (χ^2=8.25, p=0.04), while there was no effect of gland blockage on *Metarhizium* sporulation from leaf-cutting ant brood (χ^2=1.07, p=0.3; Fig. 2). A substantial number of brood of both ant species sporulated with the opportunistic fungal parasite *Aspergillus* sp. (Fig. 2). The proportion sporulating with this fungus was significantly greater when nurse ants had blocked glands, both for the weaver ants and leaf-cutting ants (respectively, χ^2=12.5, p< 0.001; χ^2=13.1, p<0.001). Few brood sporulated with *Aspergillus* when the nursing workers had functioning glands, but 48% of the weaver ant brood and 50% of the leaf-cutting ant brood did so when the glands were blocked (Fig. 2). Gland blockage therefore both significantly increased the proportion of brood exposed to *Metarhizium* that then sporulated with this parasite, and also significantly increased the proportion of brood, either treated with *Metarhizium* or not, that sporulated with opportunistic *Aspergillus* fungi.

Experiment 2: Nest hygiene

There was a significant effect of both gland blockage and ant species on the appearance of fungi on nest material, but no interaction between them (Wald=35.9, d.f.=4, p< 0.001; Wald=55.9, d.f.=1, p<0.001; Wald=5.46, d.f.=2, p=0.65 respectively). There were no significant differences between colonies (p>0.2 in both species). Both weaver ants and leaf-cutting ants experienced fungal growth sooner if one or both glands were blocked (Fig 3). For

leaf-cutting ants, compared to nest material attended by an ant with unblocked glands, the hazard ratio for nest material attended by workers with blocked metapleural glands increased to 1.4, with workers with blocked venom glands it increased to 1.99, when workers had both glands blocked it increased to 2.93, and when no worker ant was present it increased to 5.01. Blocking of the venom gland in weavers increased the hazard ratio to 2.29, and an absence of the worker ant to 2.39. Both results were significantly different ($p < 0.05$) when compared to nest silk attended to by a worker with a functional gland, but not when compared to each other, in post-hoc pairwise comparisons. Sporulation of fungi on the weaver ant silk resulted in only a sparse emergence of lightly filamentous fungi, which appeared morphologically similar across all trials and was not successfully isolated and cultured. In those leaf-cutting ant trials where the fungal crop developed other fungi, it was overgrown quickly. *Escovopsis* was found most commonly in the trials where worker ants possessed functioning glands (p=0.007; Fig 4). The appearance of *Escovopsis* was relatively lower, and of other fungi relatively higher, when the glands of the attendant workers were blocked. *Aspergillus fumigatus* was common regardless of whether the ants had functional or blocked glands, while all other fungi grew only when the fungal crop was not tended by a worker with functional glands.

Discussion

Previous work investigating the social immunity and antimicrobial secretions of ants has focused on their protection of other adults ants against parasites. The results presented here show that antimicrobial secretions produced by adult ant workers can also help increase the survival of both control and parasite-treated brood, and reduce fungal growth on nest material. Importantly, the secretions in these contexts appear to be particularly significant for

257 sanitizing against opportunistic fungi. In both leaf-cutting ants and weaver ants, and
258 regardless of experimental exposure to the *Metarhizium* parasite, brood suffered higher
259 mortality and growth of the opportunistic *Aspergillus* fungus when the workers nursing them
260 did not have functional antimicrobial-producing glands. Brood exposed to the specialist
261 fungal parasite *Metarhizium* were also more likely to sporulate with this parasite when
262 nursing workers lacked functional glands. Similarly, in both ant species, nest material was
263 more likely to be overgrown by fungi when tended by workers without functional glands.
264 This effect was most substantial in the weaver ants where blocking the venom gland was
265 sufficient to result in fungal growth on nest material comparable to when no tending ant was
266 present at all. Leaf-cutting ants required the blocking of both metapleural and venom glands
267 to show a similar result. Whilst adult insects, including ants, wasps, bees, termites and
268 eusocial thrips, utilise antibiotic secretions to protect themselves (Rosengaus et al. 2000; Bot
269 et al. 2001; Turillazzi et al. 2006; Turnbull et al. 2011; Baracchi et al. 2011, 2012), it has
270 recently been shown that *Lasius* ants transfer antimicrobial venom to enhance the resistance
271 of brood to disease (Tragust et al. 2013). Our results indicate this is also the case for
272 *Acromyrmex* and *Polyrhachis* ants. There has been much interest in the role of social
273 immunity in the disease resistance of adult social insects, but their lack of individual
274 immunity is likely to make brood the most vulnerable life-stage (Holldobler and Wilson
275 1990; Cremer et al. 2007; Gillespie et al. 1997). Social immunity may therefore be especially
276 essential for brood protection.

277 Surprisingly, there was no significant interaction in either ant species between gland
278 blockage and *Metarhizium* exposure. Antimicrobial secretions have previously been shown to
279 be very important for protecting adult leaf-cutting ants and weaver ants against exposure to
280 the *Metarhizium* parasite (Poulsen et al. 2002; Graystock et al. 2011), as well as for
281 protecting brood of *Lasius neglectus* ants (Tragust et al. 2013). The lack of a significant

282 interaction here between gland blockage and *Metarhizium* exposure is likely to be for two
283 reasons. First, both the probability of parasite infection success and the effects of
284 antimicrobial secretions are dose-dependent (Ebert et al. 2000; Hughes et al. 2004; Stow et al.
285 2007; Turnbull et al. 2012). The greater the dose of parasite, the more likely an infection is to
286 be successful, and it may be that the dose of the parasite strain used here was too high for the
287 antimicrobial secretions that were transferred from the adult ants to be fully effective in
288 defending brood against the *Metarhizium* parasite. In addition, lower doses of antimicrobial
289 compounds are less likely to be effective against a parasite and it may be that the dose of
290 antimicrobial secretions transferred to the brood was too low to fully defend the brood against
291 *Metarhizium*, and thus too low for a strong effect of gland blockage on resistance to
292 *Metarhizium* to be seen. Second, the effect of gland blockage on the mortality of even control
293 brood was relatively high. Both here and in other studies (Poulsen et al. 2002; Graystock et
294 al. 2011), there has been found to be little impact of gland blockage on control-treated adult
295 ants themselves, but it appears that control-treated brood are far more susceptible to the
296 impact of being with nursing workers with blocked glands. The behaviour of the nursing
297 workers, including their grooming of the brood, was unchanged by gland blockage, and there
298 is no known nutritional role for the glandular secretions, so it seems most probable that this
299 impact relates to the infections by opportunistic fungal parasites which developed.

300 As with all organisms, ant colonies co-exist with a wide diversity of opportunistic
301 microbes that can be parasitic, such as the *Aspergillus* fungus found in this experiment and in
302 ant colonies studied previously (Pereira and Stimac 1997; Schmid-Hempel 1998; Hughes et
303 al. 2004; Poulsen et al. 2006; Lacerda et al. 2010; Fountain and Hughes 2011). Adult ants
304 appear to suffer relatively little from these opportunistic parasites even when their production
305 of antimicrobial secretions is prevented (Poulsen et al. 2002; Graystock and Hughes 2011),
306 presumably due to their well-developed immune system and grooming behaviour. We also

found this to be the case here for brood and nest material when the antibiotic-producing glands of nurses were functioning. However, when the antimicrobial secretions of nurses were lacking, most brood succumbed to infection by opportunistic *Aspergillus* fungi and most nest material became overgrown. It cannot be excluded that some of the fungal growth may have been opportunistic growth on larvae that died from another cause. However, even if this is the case, then the results nevertheless demonstrate the importance of antimicrobial secretions from nursing workers for sanitizing the cuticles of larvae. It therefore appears that the antimicrobial secretions of adults ants are essential to protect the vulnerable brood against opportunistic parasites and to prevent nest material becoming overgrown by contaminant fungi. It may indeed be the case that the protection of larvae against ubiquitous opportunistic microbes is of greater importance for ant fitness than protection against more specialist parasites such as *Metarhizium* which tend to be rarer, and may potentially have driven the evolution of antimicrobial secretions in ants.

The leaf-cutting ant nest samples in this study were found to host at least seven species of fungi ranging from generalist, opportunistic *Aspergillus* spp. to *Escovopsis*, which specialises in parasitising the fungal crop of leaf-cutting ants (Currie 2001). Workers with functioning glands reduced both the number and diversity of fungi found compared to treatments with blocked glands. Only *Escovopsis* and the hyperabundant *Aspergillus fumigatus* (Latgé 1999) were found in treatments where the attending workers had functioning antimicrobial-producing glands. Other fungi only occurred when the fungal crop was not tended by workers with functional glands. *Escovopsis* has evolved to be highly successful in natural leaf-cutting ant nest environments (Currie 2001; Currie and Stuart 2001) and, as our results show, is able to grow on the fungal crop even when workers are producing antimicrobial compounds from their metapleural glands. In this antimicrobial-rich setting, *Escovopsis* is then able to exclude most of the opportunistic fungi found in this study.

Interestingly, however, our results suggest the specialist *Escovopsis* may be less dominant if the antimicrobial secretions of the ants are reduced, through blocking of the metapleural gland, in which setting other fungi are far more competitive against *Escovopsis*. Consequently antimicrobial secretions may be more important for protection against more opportunistic fungal pathogens than previously thought.

The results show how social immunity provided by the altruistic provision of antimicrobial secretions from adult ants has evolved to play an important role in brood survival and maintaining hygienic nest conditions, and thus the fitness of their colony. In addition, we show that these social secretions are important, not just to combat specialist parasites like *Metarhizium* and *Escovopsis*, but also in the everyday defence against opportunistic microbes which are ubiquitous in and around nest sites. This not only highlights the vulnerability of brood and nest material to disease but also their reliance on social care, and provides a compelling explanation for how immobile brood with immature immunity, survive in a world abundant with pathogens.

Acknowledgements

We thank P. Chappell, C. Frost, R. Mitchell and J. Parkinson for technical assistance, V. Norman and two anonymous reviewers for their constructive comments which improved the manuscript, IBAMA for permission to collect and export the leaf-cutting ant colonies, Martin Sebesta for providing the weaver ant colonies, and the Royal Society, BBSRC and Leverhulme Foundation for funding.

References

355 Alexander RD (1974) The evolution of social behavior. Annu Rev Ecol Syst 5:325–383

356 Andersen SB, Ferrari M, Evans HC, Elliot SL, Boomsma JJ, Hughes DP (2012) Disease
357 dynamics in a specialized parasite of ant societies. PLoS One 7:e36352

358 Armitage SAO, Boomsma JJ (2010) The effects of age and social interactions on innate
359 immunity in a leaf-cutting ant. J Insect Physiol 56:780–787

360 Baracchi D, Francese S, Turillazzi S (2011) Beyond the antipredatory defence: honey bee
361 venom function as a component of social immunity. Toxicon 58:550–557

362 Baracchi D, Mazza G, Turillazzi S (2012) From individual to collective immunity: the role of
363 the venom as antimicrobial agent in the Stenogastrinae wasp societies. J Insect Physiol
364 58:188–193

365 Baracchi D, Turillazzi S (2010) Differences in venom and cuticular peptides in individuals of
366 Apis mellifera (Hymenoptera: Apidae) determined by MALDI-TOF MS. J Insect
367 Physiol 56:366–375

368 Blum MS (1992) Ant venoms: chemical and pharmacological properties. Toxin Rev 11:115–
369 164

370 Boomsma JJ, Schmid-Hempel P, Hughes WOH (2005) Life histories and parasite pressure
371 across the major groups of social insects. In: Fellowes M, Holloway G, Rolff J (eds.)
372 Insect Evolutionary Ecology.CABI Publishing, Wallingford, UK 139–176

373 Bot ANM, Obermayer ML, Hölldobler B, Boomsma JJ (2001) Functional morphology of the
374 metapleural gland in the leaf-cutting ant *Acromyrmex octospinosus*. Insectes Soc 48:63–
375 66

376 Bulet P, Hetru C, Dimarcq J-L, Hoffmann D (1999) Antimicrobial peptides in insects;
377 structure and function. Dev Comp Immunol 23:329–344

378 Chapuisat M, Oppliger A, Magliano P, Christe P (2007) Wood ants use resin to protect
379 themselves against pathogens. Proc R Soc Lond B 274:2013–2017

380 Cremer S, Armitage SAO, Schmid-Hempel P (2007) Social immunity. Curr Biol 17:693–702

381 Currie CR (2001) A community of ants, fungi, and bacteria: a multilateral approach to
382 studying symbiosis. Annu Rev Microbiol 55:357–380

383 Currie CR, Mueller UG, Malloch D (1999) The agricultural pathology of ant fungus gardens.
384 Proc Natl Acad Sci USA 96:7998–8002

385 Currie CR, Stuart AE (2001) Weeding and grooming of pathogens in agriculture by ants.
386 Proc R Soc Lond B 268:1033–1039

387 de Souza AL, Soares IMF, Cyrino LT, Eduardo Serro J (2006) The metapleural gland in two
388 subspecies of *Acromyrmex subterraneus* (Hymenoptera: Formicidae). Sociobiology
389 47:19–25

390 Dornhaus A, Powell S, Bengston S (2010) Group size and its effects on collective
391 organization. Annu Rev Entomol 57:123–141

392 Ebert D, Zschokke-Rohringer CD, Carius HJ (2000) Dose effects and density-dependent
393 regulation of two microparasites of *Daphnia magna*. Oecologia 122:200–209

394 Evans HC, Elliot SL, Hughes DP (2011) Hidden diversity behind the zombie-ant fungus
395 *Ophiocordyceps unilateralis*: four new species described from carpenter ants in Minas
396 Gerais, Brazil. PLoS One 6:e17024

397 Evans JD, Aronstein K, Chen YP, Hetru C, Imler JL, Jiang H, Kanost M, Thompson GJ, Zou
398 Z, Hultmark D (2006) Immune pathways and defence mechanisms in honey bees *Apis*
399 *mellifera*. Insect Mol Biol 15:645–656

400 Fernández-Marín H, Zimmerman JK, Rehner SA, Wcislo WT (2006) Active use of the
401 metapleural glands by ants in controlling fungal infection. Proc R Soc Lond B
402 273:1689–1695

403 Foley K, Fazio G, Jensen AB, Hughes WOH (2012) Nutritional limitation and resistance to
404 opportunistic *Aspergillus* parasites in honey bee larvae. J Invertebr Pathol 111:68–73

405 Fountain T, Hughes WOH (2011) Weaving resistance: silk and disease resistance in the
406 weaver ant *Polyrhachis dives*. Insectes Soc 58:453-458

407 Gillespie JP, Kanost MR, Trenczek T (1997) Biological mediators of insect immunity. Annu
408 Rev Entomol 42:611–643

409 Graystock P, Hughes WOH (2011) Disease resistance in a weaver ant, *Polyrhachis dives*, and
410 the role of antibiotic-producing glands. Behav Ecol Sociobiol 65:2319-2327

411 Haine ER, Moret Y, Siva-Jothy MT, Rolff J (2008) Antimicrobial defense and persistent
412 infection in insects. Science 322:1257–1259

413 Hamilton C, Lejeune BT, Rosengaus RB (2011) Trophallaxis and prophylaxis: social
414 immunity in the carpenter ant *Camponotus pennsylvanicus*. Biol Lett 7:89–92

415 Henry T, Iwen P, Hinrichs S (2000) Identification of *Aspergillus* species using internal
416 transcribed spacer regions 1 and 2. J Clin Microbiol 38:1510-1515

417 Hölldobler B, Wilson EO (1990) The ants. Belknap Press, Cambridge

418 Hughes WOH, Bot ANM, Boomsma JJ (2010) Caste-specific expression of genetic variation
419 in the size of antibiotic-producing glands of leaf-cutting ants. Proc R Soc Lond B
420 277:609–615

421 Hughes WOH, Eilenberg J, Boomsma JJ (2002) Trade-offs in group living: transmission and
422 disease resistance in leaf-cutting ants. Proc R Soc Lond B 269:1811–1819

423 Hughes WOH, Pagliarini R, Madsen HB, Dijkstra MB, Boomsma JJ (2008) Antimicrobial
424 defense shows an abrupt evolutionary transition in the fungus-growing ants. Evolution
425 62:1252–1257

426 Hughes WOH, Petersen KS, Ugelvig L V, Pedersen D, Thomsen L, Poulsen M, Boomsma JJ
427 (2004) Density-dependence and within-host competition in a semelparous parasite of
428 leaf-cutting ants. BMC Evol Biol 4:45–57

429 Hughes WOH, Thomsen L, Eilenberg J, Boomsma JJ (2004) Diversity of entomopathogenic
430 fungi near leaf-cutting ant nests in a neotropical forest, with particular reference to
431 *Metarhizium anisopliae* var. *anisopliae*. J Invertebr Pathol 85:46–53

432 Keller S, Kessler P, Schweizer C (2003) Distribution of insect pathogenic soil fungi in
433 Switzerland with special reference to *Beauveria brongniartii* and *Metharhizium*
434 *anisopliae*. BioControl 48:307–319

435 Konrad M, Vyleta ML, Theis FJ, Stock M, Tragust S, Klatt M, Drescher V, Marr C, Ugelvig
436 LV, Cremer S (2012) Social transfer of pathogenic fungus promotes active
437 immunisation in ant colonies. PLoS Biology 10:1-15

438 Krause J, Ruxton GD (2002) Living in groups. Oxford University Press, New York

439 Kuhn-Nentwig L (2003) Antimicrobial and cytolytic peptides of venomous arthropods. Cell
440 Mol Life Sci 60:2651–2668

441 Latgé J (1999) *Aspergillus fumigatus* and Aspergillosis. Clin Microbiol Rev 12:310-350

442 Lavine MD, Strand MR (2002) Insect hemocytes and their role in immunity. Insect Biochem
443 Mol Biol 32:1295–1309

444 Little AEF, Murakami T, Mueller UG, Currie CR (2006) Defending against parasites:
445 fungus-growing ants combine specialized behaviours and microbial symbionts to protect
446 their fungus gardens. Biol Lett 2:12–16

447 Mackintosh JA, Trimble JE, Beattie AJ, Veal DA, Jones MK, Karuso PH (1995)
448 Antimicrobial mode of action of secretions from the metapleural gland of *Myrmecia*
449 *gulosa* (Australian bull ant). Can J Microbiol 41:136–144

450 Mendonça ADL, Silva CE da, Mesquita FLT de, Campos RDS, Nascimento RR Do, Ximenes
451 ECPDA, Sant'Ana AEG (2009) Antimicrobial activities of components of the glandular
452 secretions of leaf cutting ants of the genus *Atta*. Antonie Van Leeuwenhoek 95:295–303

453 Milner RJ, Staples JA, Lutton GG (1998) The selection of an isolate of the Hyphomycete
454 fungus, *Metarhizium anisopliae*, for control of termites in Australia. Bio Control
455 247:240–247

456 Morelos-Juárez C, Walker TN, Lopes JFS, Hughes WOH (2010) Ant farmers practice
457 proactive personal hygiene to protect their fungus crop. Curr Biol 20:553–554

458 Mueller U, Rehner S, Schultz T (1998) The evolution of agriculture in ants. Science 281:
459 2034–2038

460 Pereira R, Stimac J (1997) Biocontrol options for urban pest Ants. J Agric Entomol 14:231–
461 248

462 Poulsen M, Bot ANM, Nielsen MG, Boomsma JJ (2002) Experimental evidence for the costs
463 and hygienic significance of the antibiotic metapleural gland secretion in leaf-cutting
464 ants. Behav Ecol Sociobiol 52:151–157

465 Poulsen M, Hughes WOH, Boomsma JJ (2006) Differential resistance and the importance of
466 antibiotic production in *Acromyrmex echinatior* leaf-cutting ant castes towards the
467 entomopathogenic fungus *Aspergillus*. Insectes Soc 53:349-355

468 Reber A, Chapuisat M (2011) Diversity, prevalence and virulence of fungal entomopathogens
469 in colonies of the ant *Formica selysi*. Insectes Soc 59:231-239

470 Reber A, Purcell J, Buechel SD, Buri P, Chapuisat M (2011) The expression and impact of
471 antifungal grooming in ants. J Evol Biol 24:954–964

472 Rosengaus RB, Maxmen AB, Coates LE, Traniello JFA (1998) Disease resistance: a benefit
473 of sociality in the dampwood termite *Zootermopsis angusticollis* (Isoptera:
474 Termopsidae). Behav Ecol Sociobiol 44:125–134

475 Rosengaus RB, Lefebvre ML, Traniello JFA (2000) Inhibition of fungal spore germination by
476 *Nasutitermes*: evidence for a possible antiseptic role of soldier defensive secretions. J
477 Chem Ecol 26:21–39

478 Rosengaus RB, Traniello JFA, Lefebvre ML, Maxmen AB (2004) Fungistatic activity of the
479 sternal gland secretion of the dampwood termite *Zootermopsis angusticollis*. Insectes
480 Soc 51:1-6

481 Schmid-Hempel P (1998) Parasites in social insects. Princeton University Press, Princeton

482 Storey GK, Meer RK Vander, Boucias DG, McCoy CW (1991) Effect of fire ant (*Solenopsis*
483 *invicta*) venom alkaloids on the in vitro germination and development of selected
484 entomogenous fungi. J Invertebr Pathol 58:88–95

485 Stow A, Beattie A (2008) Chemical and genetic defences against disease in insect societies.
486 Brain Behav Immun 22:1009-1013

487 Stow A, Briscoe D, Gillings M, Holley M, Smith S, Leys R, Silberbauer T, Turnbull C,
488 Beattie A (2007) Antimicrobial defences increase with sociality in bees. Biol Lett
489 3:422–424

490 Sumner S, Hughes WOH, Boomsma JJ (2003) Evidence for differential selection and
491 potential adaptive evolution in the worker caste of an inquiline social parasite. Behav
492 Ecol Sociobiol 54:256–263

493 Tragust S, Mitteregger B, Barone V, Konrad M, Ugelvig L V, Cremer S (2013) Ants disinfect
494 fungus-exposed brood by oral uptake and spread of their poison. Curr Biol 23:76-82

495 Traniello JFA, Rosengaus RB, Savoie K (2002) The development of immunity in a social
496 insect: Evidence for the group facilitation of disease resistance. Proc Natl Acad Sci USA
497 99:6838–6842

498 Turillazzi S, Mastrobuoni G, Dani FR, Moneti G, Pieraccini G, Marca G la, Bartolucci G,
499 Perito B, Lambardi D, Cavallini V, Dapporto L (2006) Dominulin A and B: two new
500 antibacterial peptides identified on the cuticle and in the venom of the social paper wasp
501 *Polistes dominulus* using MALDI-TOF, MALDI-TOF/TOF, and ESI-ion trap. J Am Soc
502 Mass Spectrom 17:376–383

503 Turnbull C, Caravan H, Chapman T, Nipperess D, Dennison S, Schwarz M, Beattie A (2012)
504 Antifungal activity in thrips soldiers suggests a dual role for this caste. Biol Lett 8:526–
505 529

506 Turnbull C, Hoggard S, Gillings M, Palmer C, Stow A, Beattie D, Briscoe D, Smith S,
507 Wilson P, Beattie A (2011) Antimicrobial strength increases with group size:
508 implications for social evolution. Biol Lett 7:249–252

509 Ugelvig LV, Cremer S (2007) Social prophylaxis: group interaction promotes collective
510 immunity in ant colonies. Curr Biol 17:1967–1971

511 Walker TN, Hughes WOH (2009) Adaptive social immunity in leaf-cutting ants. Biol Lett
512 5:446–448

513 Wilson-Rich N, Spivak M, Fefferman NH, Starks PT (2009) Genetic, individual, and group
514 facilitation of disease resistance in insect societies. Annu Rev Entomol 54:405–423

515 Zenghe W (1986) A preliminary study of the ant, *Polyrhachis dives* F. Smith. Sci Silvae Sin
516 4:15

517 Zelezetsky I, Pag U, Antcheva N, Sahl H-G, Tossi A (2005) Identification and optimization
518 of an antimicrobial peptide from the ant venom toxin pilosulin. Arch Biochem Biophys
519 434:358–364

520

Figure legends

Fig. 1 Survival of a) weaver ant pupae and b) leaf-cutting ant larvae that were treated with either *Metarhizium* parasite (solid lines) or control solution (dashed lines) and cared for by workers either with (open circles) or without (black circles) functional antimicrobial glands (the venom gland for weaver ants and the metapleural gland for leaf-cutting ants). For each species, different letters indicate treatments which differed significantly from one another at $P < 0.05$ in pairwise comparisons with Kaplan-Meier tests.

Fig. 2 Proportions of a) weaver ant larvae and b) leaf-cutting ant pupae that produced conidia of the *Metarhizium* experimental parasite (black), the opportunistic *Aspergillus* fungus (grey), or remained uninfected (white). Brood were either treated with *Metarhizium* parasite or control solution, and kept with workers either with or without functional antibiotic-producing glands (the venom gland for weaver ants and the metapleural gland for leaf-cutting ants).

Fig. 3 Proportion of a) weaver ant silk material and b) leaf-cutting ant fungal crop material that was free of contaminant fungal growth when cared for by workers with functional glands (white circles), blocked venom gland (black circles), blocked metapleural gland (black diamonds), both glands blocked (black squares), or where the worker ant was absent (dashed line). For each species, different letters indicate treatments which differed significantly from one another at $P < 0.05$ in pairwise comparisons with Kaplan-Meier tests.

Fig. 4 Proportion of trials where foreign fungus overgrew leaf-cutting ant nest material grouped by treatment. Foreign fungal species were *Aspergillus fumigatus* (white), *Aspergillus*

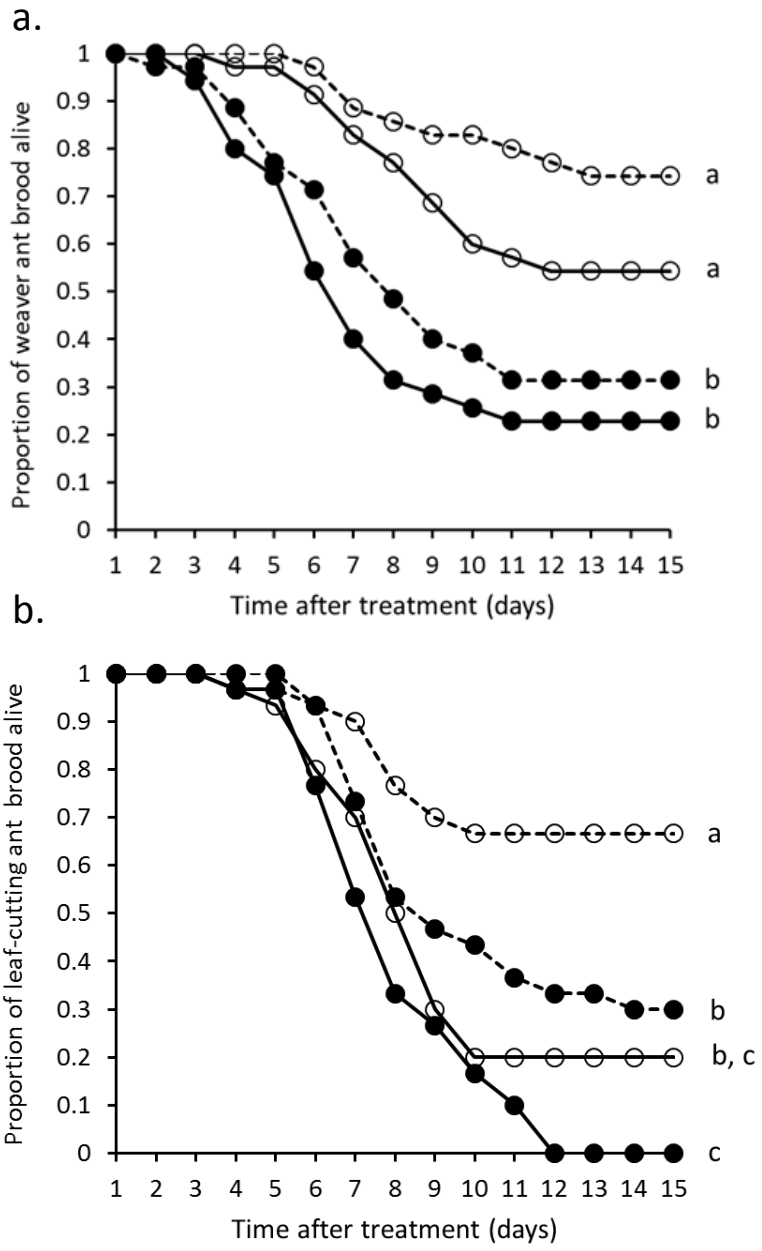
544 *tamarii* (light grey), *Aspergillus nomius* (dark grey), *Aspergillus sclerotiorum* (black),

545 *Fusarium* sp. (leftward diagonals), *Trichoderma* sp. (cross-hatched), *Escavopsis* sp.

546 (rightward diagonals).

547

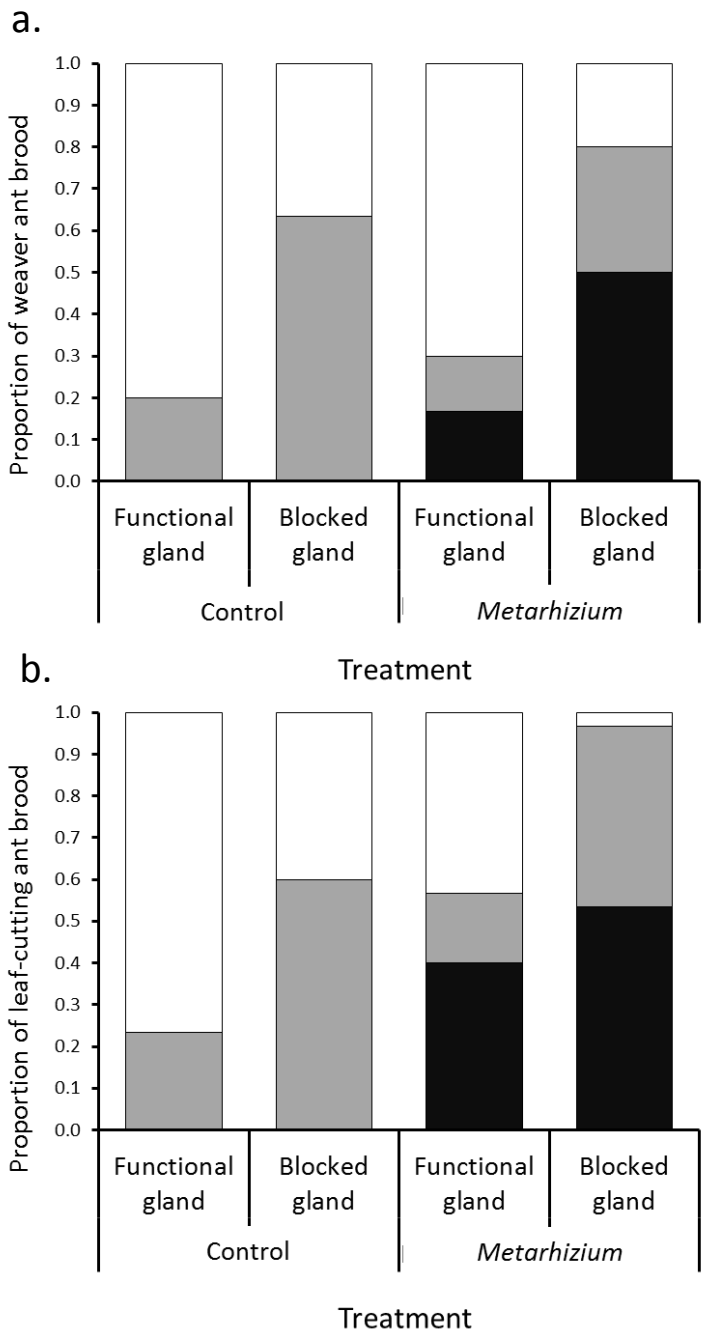
548 Fig. 1



549

550

551 Fig. 2.

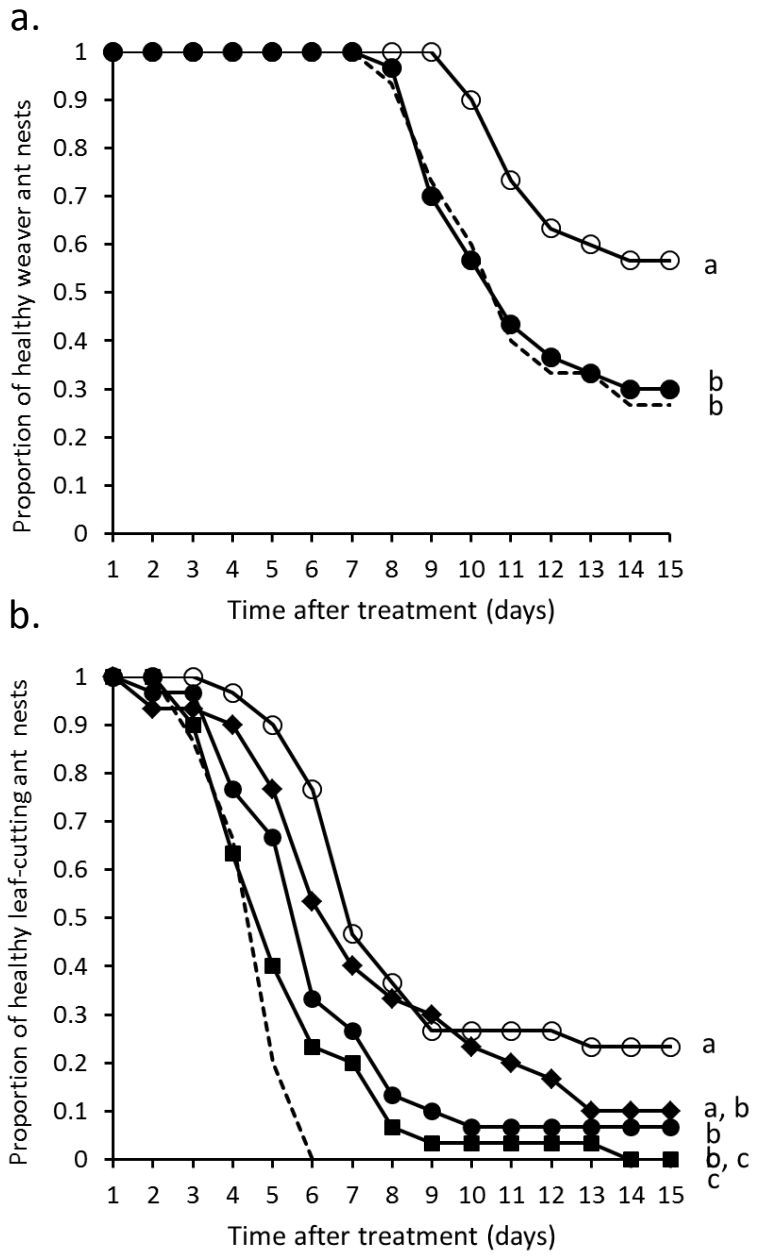


552

553

554

555 Fig. 3.

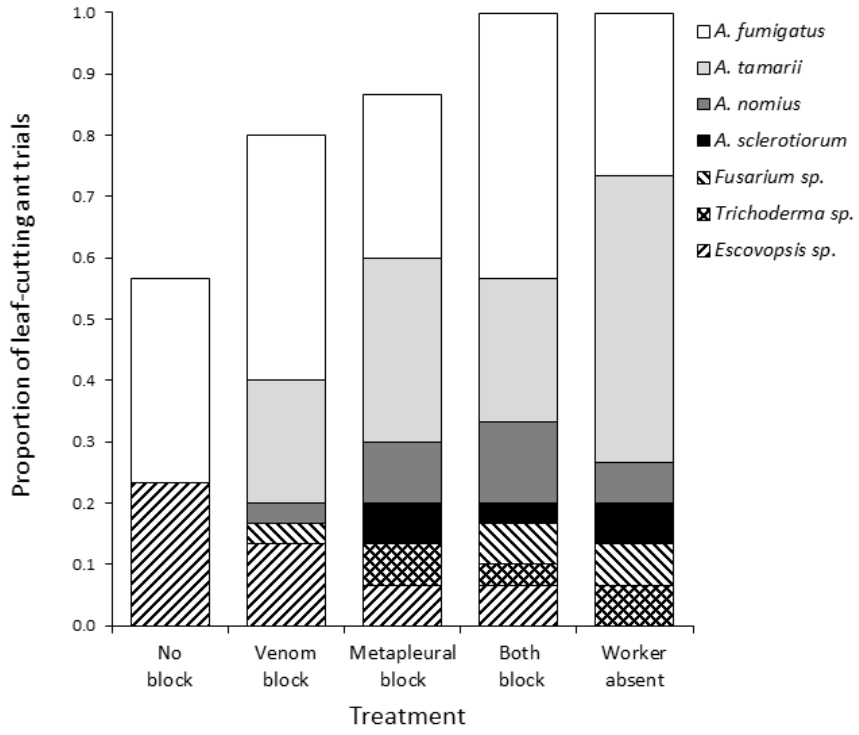


556

557

558

559 Fig 4.



560

561

Supporting Information

S1_exp1methods_suppinfo.tif

S2_exp2methods_suppinfo.tif

S3_nursebehaviours_suppinto

S1. Details of treatment structure and subject assignment for Experiment 1. 120 leaf-cutting ants (a) were used in total, split between two equal cohorts. Each of the cohorts consisted of 20 ants from each of the three colonies, giving a total of 60 ants for each cohort. Within each cohort, ants from individual colonies were divided evenly into four treatment groups, consisting of five ants. This gave a total of 30 replicate ants per treatment across all colonies and cohorts. 160 weaver ants (b) were used in total, split between two equal cohorts. Each of the cohorts consisted of 40 ants from each of the two colonies, giving a total of 80 ants for each cohort. Within each cohort, ants from individual colonies were split evenly into four treatment groups, consisting of 10 ants. This gave a total of 40 replicate ants per treatment across all colonies and cohorts.

S2. Details of treatment structure and subject assignment for Experiment 2. 120 leaf-cutting ants (a) were used in total, consisting of 40 ants from each of the three colonies. Ants from individual colonies were divided evenly into four treatment groups, consisting of 10 ants. This gave a total of 30 replicate ants per treatment across all colonies. Fifteen additional blank trials were conducted in the absence of any ant. 60 weaver ants (b) were used in total, consisting of 30 ants from each of the two colonies. Ants from individual colonies were divided evenly into two treatment groups, consisting of 15 ants each. This gave a total of 30

585 replicate ants per treatment across all colonies. Fifteen additional blank trials were conducted
586 in the absence of any ant.

587

588 **S3. Results of experiment comparing brood-care behaviour and survival of nurse ants**
589 **with blocked and unblocked glands**. Both leaf-cutting ants (a; *Wald*=5.6, *d.f.*=1, *p*=0.45) and
590 weaver ants (b; *Wald*=2.1, *d.f.*=1, *p*=0.15) showed no difference in survival of nurses with
591 (open circles) or without (black circles) functional antimicrobial glands, whilst caring for
592 brood treated with either *Metarhizium* parasite (solid lines) or control solution (dashed lines),
593 over the course of the experiment. Additionally neither leaf-cutting ants (c) or weaver ants (d)
594 showed any differences in the duration of time spent interacting with brood (*U*=39, *d.f.*=9,
595 *z*=0.84, *p*=0.4, and *U*=41.5, *d.f.*=9, *z*=0.64, *p*=0.52, respectively), the incidences of contact with
596 brood (*U*=46, *d.f.*=9, *z*=0.36, *p*=0.72, and *U*=43, *d.f.*=9, z=0.54, *p*=0.59, respectively), or incidences
597 of brood-grooming (*U*=49.5, *d.f.*=9, *z*=0.54, *p*=0.96, and *U*=46.5, *d.f.*=9, *z*=0.27, *p*=0.79,
598 respectively), between nurse ants with blocked (dark bars) and unblocked glands (light bars).

599